Applicant has made reference to these paragraph numbers to facilitate the review of Applicant's response.

4a The Examiner has withdrawn the enablement rejection of claim 20, pursuant to Applicant's previous amendment.

4b The other enablement rejections are maintained. These rejections arise from the disbelief that applicant's invention actually works, as for example, stated on page 3 lines 15-16 of the first Office Action "transfer [of myoblasts in humans] has not been successful and is at best controversial." The Examiner has thus argued that the invention is inoperable and that (see page 4 top from the first Office Action) "the art teaches that human transfer has not been successful or at best that the results of human transfer have been controversial" noting the entire document of Hoffman, a scientist peer.

Applicant responds that before his effort, transfer by others in humans was not successful. Since the 1993 publication date of Hoffman, the pioneering significance of applicant's work described in the present application has become more appreciated, even by Hoffman. Evidence of this is a more recent apology by Dr. Eric Hoffman, a copy of which is attached as Appendix A. This gradual turn around in the last 7 years has occurred through applicant's diligent effort, using the large numbers of cells in the procedure as described. In fact, using the procedure, both the FDA and scientific peers have come to accept the reality that MTT works. The FDA has reviewed and has formally accepted the procedure for further development in the United States. A copy of the formal acceptance is attached as Appendix B.

The field generally accepts MTT, as evidenced by other more recent publications in peer-reviewed journals. This success has come from applicant's laboratory using the described procedures. Applicant provides two such publications in Appendix C, and points out that no particular new techniques were required other than that described in the present specification.

Applicant emphasizes the need to implant or inject large numbers of cells. In fact, using the large numbers described in the specification, a human recipient undergoes an alteration in cosmetic appearance. Applicant was surprised to find striking changes in such appearances, and from those observations, further appreciated the cosmetic significance of MTT using large numbers of cells. A representative figure of a cosmetic change is shown in the figure of Appendix D, attached, which displays bulking up of an arm from MTT. These data dramatically show the cosmetic effect of MTT in humans and underscores the successful use of the procedure taught in the specification. Removal of the rejection is respectfully requested in light of these data and acceptance by others.

Applicant further points out that applicant initially obtained data with mice, using the methodology described in the specification and that the Patent Office accepts data from animal testing to show therapeutic utility. In particular, MPEP § 2107.01 provides that data generated from testing in an animal model almost invariably will be sufficient to establish therapeutic utility for a process, if the data is reasonably correlated to the particular therapeutic utility. The Examiner has not provided a scientific basis for dismissing any of the procedural steps (for example, from page 24, line 26 through page 26, line 19) taught as being relevant only for the mouse, or for concluding that the procedure, while enabled for mice, cannot be scientifically applied to the human. In the absence of such concrete solid science, the assertion of enablement in the human must be accepted and applicant respectfully requests removal of this rejection.

Applicant has used the described MTT procedures in humans. Specific details describing such use are presented below, emphasizing how the method differs from previous methods, allowing for success in mammals generally:

(1) The specification teaches procedures for injecting myoblast cells into tissues *in vivo*. These procedures are distinct from and represent advances over techniques previously used. For example, Fig. 6 illustrates that a diagonal two-inch injection into a host promotes maximum myoblast fusion. Another distinct technique is the study of myofiber orientation to determine transverse injection. Yet another distinct technique is the injection of "billions" exemplified with injecting 12.5 billion and 30 billion myoblast cells

into humans to induce cell fusion as described in the specification on page 12, lines 8 and 33. Applicant has already pointed out the need to inject such large numbers because of his appreciation that others failed partly because they lacked sufficient cell number (See Applicant's response to First Office Action, page 5, lines 15-17).

The publications that the Examiner relies on, however, disclose injecting considerably less myogenic cells (i.e. 10^6-10^9), while failing to assess the importance of the angle of injection. This is a particularly salient point for cosmetic treatment because large numbers of cells make a larger difference. Applicant, for the record, provides an explanation for the failure of others to obtain successful MTT in human beings. Elevated levels of myoblasts are needed to compensate for degradation by macrophages during the first three weeks after transfer as described in Law *et al. Gene Ther. Mol. Bio.* Vol. 1, 345-363 (1998) and in Law *et al. Cell Tran.* Vol. 6, No. 1 (1997). Because of this insight, applicant uses sufficient numbers of injected myoblasts to satisfy the degradation and has achieved success. The Examiner has not provided a prior art reference that addresses this important limitation, in making the enablement rejection. To highlight the importance of this difference, applicant has added the term "an effective amount" to independent claim 20 and the claims dependent thereon. The specification indicates that such an effective amount involves billions of cells. See, for example, page 12 top, (describing use of 12.5 billion myoblasts) and page 12 bottom, which describes the use of 30 billion myoblasts.

(2) The specification provides data for injection of myoblasts into a number of different muscles in boys with Duchenne muscular dystrophy (DMD) and infantile facioscapulohumeral dystrophy (IFSH). (See SUMMARY OF THE INVENTION, page 11, first paragraph). Results from these studies show that the MTT procedure as described in the specification, when using a sufficient number of cells, works in humans. See the specification for example, from page 12, line 33 to page 13, line 3. The Examiner has not provided a reason for dismissing this data as false or for dismissing applicant's technique. If the Examiner for some reason believes that information in the patent specification is false, the Examiner is specifically requested to point out that information.

(3) By using the procedure taught in the specification, applicant has obtained successful results for MTT in humans. The Food and Drug Administration (FDA) has

recognized such success by designating Applicant's related Investigational New Drug Application (IND) as a Fast Track Product, as described above. The PTO properly cannot deem a procedure unenabled after that procedure has been reviewed and accepted by the FDA for clinical trials. The Examiner respectfully is requested to defer to the FDA's judgement and to remove the enablement rejection based on the Examiner's belief that MTT does not work.

The technique of treating degenerative diseases such as DMD *via* MTT and the technique of augmenting body tissue for cosmetic purposes *via* MTT both rely on administering myogenic cells into the body to generate muscle growth. The specification discloses sufficient information for a skilled artisan to practice MTT for both purposes. The Examiner has argued based on Coovert *et al.* and Hoffman (see First Office Action, page 4, lines 6-7) that MTT in humans is inoperable. For reasons presented in the earlier response, Applicant addresses this argument in the sections that follow.

4c The Examiner relies on the teaching in Dimaro *et al.* to state that "it is unclear how donor myoblasts could be used to alter the appearance of non-diseased muscle." But Dimaro contains no teaching that incorporation of donor myoblasts in normal uninjured or non-diseased muscle is impossible. Instead, Dimaro only states that "regenerating muscle provides a better environment for myoblast transfer and incorporation into new and existent muscle fibers than gwowing musculature" (Dimaro *et al.* at 333). Dimaro *et al.*, therefore, infers that incorporation of myoblasts into uninjured and non-diseased muscle *does* occur, albeit not as much in injured or regenerating muscle tissue.

The claimed invention does not require a particular degree to which donor myoblasts contribute to muscle formation. That is, "augmentation" does not require 100% contribution of donor myoblasts. For example, claims 21 and 30 do not require that a specific percentage of donor myogenic cells fuse with myoblasts. They only require that *some* fusion must occur. Thus, the "degree to which myoblasts contribute to new muscle formation" (paragraph "4c," page 5, lines 10-11) is irrelevant.

The Examiner has questioned "how administration of myoblasts to breast tissue (which is composed of adipose tissue) or to a hip (which is composed of bone) could be used to alter the cosmetic appearance of the breast or hip." (First Office Action, p. 5, lines

4-6). As stated in the earlier response, such alteration in cosmetic appearance arises from fusion of donor myoblasts with myoblasts in the tissue. Breast tissue comprises more than just "adipocytes," as the Examiner seems to imply. (First Office Action, p.5, line 7). In fact, breast tissue contains myoblasts, which are the cells that fuse with the myogenic composition. Applicant has amended claims 21 and 30 to better clarify the fact that myoblasts in the recipient tissue area primarily are responsible for fusing with donor myoblasts.

4(d) Applicant disagrees with the Examiner's position that "one of skill in the art would be unable to [inject proliferating undifferentiated muscle cells into humans without tumor formation at the injection site]." (*See* First Office Action, paragraph "4d," page 6, lines 1-2). In particular, the specification discloses a method for avoiding tumor production by limiting proliferation to no more than "30 generations." (*See* page 24, lines 31-34). Applicant does acknowledge the Examiner's position that claim 20 as filed includes immunocompromised hosts. In order to expedite prosecution, claim 20 has been amended.

Additional Grounds of Rejection under §112:

The Examiner has rejected newly added claims 31 and 32 under 35 U.S.C. 112, first paragraph. As the basis for the rejection, the Examiner states, "[t]he art teaches that surgical procedures are performed so as to minimize trauma to the patient," and that surgical implants can be performed without dissecting a "body part" (*sic*). The Examiner then concludes, "[g]iven these teachings, it is difficult to reconcile dissection [of tissue] as a viable means of augmenting a body part."

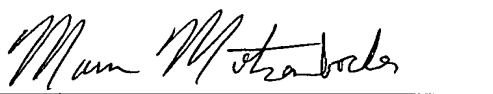
A section 112 first paragraph rejection is improper because none of the reasons provided for the rejection concerns enablement. Instead, the Examiner infers that the claimed method would be more traumatic than other known methods of augmentation. The Examiner has overlooked the significance of the specification in this context, which for example states on page 28, lines 11-13: "Since muscles can develop great forces and scar tissues are inert, the developing muscles will force their scar tissues aside throughout their existence." Even if one could argue that myotube transfer is more traumatic than other types of augmentation procedures, it is not proper to use such an argument as a basis for an enablement rejection under section 112, first paragraph. One of many possible applications

of this method would be to stimulate growth of a body part, after a tumor has been removed by implanting myotubes into the space created by tumor removal. Applicant has amended claim 31 by adding this step. In addition, Applicant has added claim 33 to better clarify the application of this method.

The Examiner also has rejected claims 31 and 32 under 35 U.S.C. 112, second paragraph, as being incomplete for omitting an essential step. However, the claimed method removes tissue from a body part and implants myotubes into the space created by this removal. There is no implantation of myotubes into the removed tissue and the claim is not interpreted that way. Nor is the removed tissue reinserted into the body part after dissection. To construe the claim otherwise would ignore the plain language of the claim. Therefore, reconsideration and removal of this rejection are respectfully requested.

Reconsideration and allowance are, accordingly, solicited.

Respectfully submitted,



February 7, 2000
Date

Marvin A. Motsenbocker
Reg. No. 36,614

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Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No. 19-0741 for any such fees; and applicant(s) hereby petition for any needed extension of time.

Appendix A

University of Pittsburgh

School of Medicine
Department of Molecular Genetics and Biochemistry
Biomedical Science Tower, Room E12-40

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412-648-9570
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Letter of Retraction and Apology which reads as follows:

On several occasions, I have made damaging statements regarding Dr. Peter K. Law and Cell Therapy Research Foundation of Memphis, Tennessee. These statements were made to members of the media and were carried over the University of Pittsburgh's internet site. Specifically, I made statements that impugned the personal character and scientific integrity of Dr. Law and Cell Therapy Research Foundation. I apologize publicly to Dr. Law and Cell Therapy Research Foundation for these statements and I hereby retract them. I wish Dr. Law and Cell Therapy Research Foundation the best of success in their efforts to find a treatment for muscular dystrophy with myoblast transfer therapy.

Eric P Hoffman, PhD

2/2/99
Date

Appendix B

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

OCT 16 1998

Our Reference: BB-IND 5108

Cell Therapy Research Foundation
Attention: Peter K. Law, Ph.D., Chairman
1770 Moriah Woods Boulevard, Suite 18
Memphis, TN 38117

Dear Dr. Law:

Reference is made to your **Investigational New Drug Application (IND)** for "Cultured Allogeneic Myoblasts. and Cyclosporin (Sandoz)." We also refer to your submission of August 18, 1998, received on August 19, 1998, requesting designation as a Fast Track Product pursuant to Section 506 of the Food, Drug, and Cosmetic Act (the Act).

We have reviewed your request and we are designating as a Fast Track development program the investigation of cultured allogeneic myoblasts for delay or prevention of severe disability and/or death in patients with Duchenne Muscular Dystrophy.

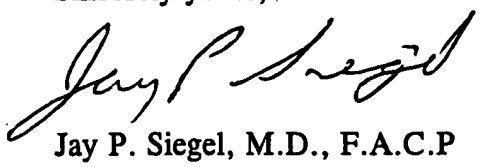
Please note that if the clinical development program you pursue does not continue to meet the criteria for Fast Track designation, the application will not be reviewed under the Fast Track program.

Under the FDA Modernization Act of 1997, designation as a Fast Track product for a new drug or biological product means that FDA will take such actions as are appropriate to expedite the development and review of the application for approval of such product. FDA may also evaluate for filing and commence review of portions of an application for approval of a Fast Track product under certain conditions.

FDA is the process of preparing detailed guidance on the provisions of Section 112 of the FDA Modernization Act of 1997 as required by the Act. Until such time as the guidance is publicly available, please contact our office in order to receive guidance on the development and review of your product and how these provisions will be applied. We look forward to working with you to expedite the development and review of this promising proposed use of the product.

If you any have questions, please contact Jeanne Delasko, Division of Application Review and Policy, at (301) 827-5101.

Sincerely yours,



Jay P. Siegel, M.D., F.A.C.P
Director
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

Appendix C

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Gene Ther Mol Biol Vol 1, 345-363. March, 1998.

Myoblast transfer as a platform technology of gene therapy

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Keywords: Myoblast transfer; Clinical trials; Gene therapy; Duchenne muscular dystrophy; Viral vectors

Summary

Myoblasts divide profusely, and fuse during muscle regeneration, interiorizing MHC-I antigens and inserting myonuclei with the normal genome into muscles of genetically deficient recipients, where any replacement gene can be stably integrated and naturally expressed. Myoblasts are the natural source and vehicle for many gene therapies. Myoblast transfer therapy is completing US FDA Phase II clinical trials for Duchenne muscular dystrophy.

I. Introduction

The National Institute of Standards and Technology has recently announced that tissue engineering will likely be the key to treating genetic diseases and degenerative disorders that accounted for 50% of the $1+ trillion U.S. health care cost in 1995 (Schwartz, 1997; Langer and Vacanti, 1993; Nerem and Sambanis, 1995).

Among the many programs of tissue engineering, gene therapy has been hailed as the medicine of the 21st century. Despite the nearly universal belief that gene therapy will ultimately allow the treatment of currently incurable diseases and conditions, its potential remains largely unfulfilled (Hillman et al., 1996). Only when a safe and effective gene delivery technology has been proven in humans can the full potential of gene therapy be realized.

To date, over 3000 subjects worldwide have received gene therapies among the 280+ protocols approved. Data indicate that no single vector will serve all systems. In examining gene transfer methods mediated by particle bombardment (Jiao et al., 1993; Sautter et al., 1991), liposomes (Stewart et al., 1992; Ray and Gage, 1992), calcium phosphate precipitation (Ray and Gage, 1992; Albert and Tremblay, 1992), and electroporation (Ray and Gage, 1992; Albert and Tremblay, 1992; Puchalski and Fahl, 1992), one can conclude that transduction efficiency is extremely low and variable. The level of transgene expression depends on the promoter strength in a particular cell type. Only liposomes, together with retroviruses, adenoviruses, adeno-associated viruses and myoblasts have been used in clinical trials.

A. Vectors

1. Liposomes

Cationic liposome/DNA complexes gain cellular entry via receptor-mediated endocytosis (Stewart et al., 1992; Trubetskoy et al., 1992). Assuming the transgene escapes digestion by the endosome, it has no built-in mechanism to get across the nuclear membrane and is therefore non-integrative. The minimal and transient expression of the transgene is the result of random targeting, integration, and regulation. Liposomes have the advantage of being non toxic and can therefore be used in large quantities and repeatedly (Brenner, 1995).

2. Viruses

The viral vectors were the first to gain widespread scientific applications. Notable was "the first federally approved gene therapy protocol, for correction of adenosine deaminase (ADA) deficiency, began on 14 September 1990"(Anderson, 1990, 1992, 1995).

Retroviral vectors can transduce dividing cells with integration into host DNA. They integrate randomly and may cause mutation and cell death. They exhibit no toxicity. Although they can house larger transgenes than adenoviruses and adeno-associated viruses, the capacity is less than 10 kb. They are unstable in primate complement and cannot be targeted to specific cell types *in vivo* (Brenner, 1995; Cornetta et al., 1991).

Adeno-associated viruses and adenoviruses have shown considerable promise and are widely used. They can accommodate a broad range of genetically modified genes; are efficiently taken up by non-dividing cells *in vivo*; do not integrate into chromosomal DNA, thus reducing the risk of insertional mutagenesis; and are amenable to redirected tissue targeting (Morsey and Caskey, 1997).

All viruses can cause harm when they revert to wild type and become replication-competent (Brenner, 1995; Coutelle et al., 1994; Curiel et al., 1996). Dose-dependent inflammation occurred after nasal (Knowles et al., 1995) or lung (Crystal et al., 1994) administration of the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA conjugated with adenoviral vectors. The low efficacy, if any, is what one would have expected of pioneering studies. However, the risk to benefit ratio cannot be ignored. Also viruses produce antigens. When exposed to the host immune system, through leakage, secretion or cell damage, these antigens trigger immune reactions against the transduced cells. Certain viral elements are also toxic. These three inherent problems post almost insurmountable difficulties that prohibit the safe and efficacious clinical use of viral vectors at the present except for terminal cases. To raise caution, the FDA has mandated viral vector validation of every batch to be used on humans.

3. Plasmids

Single gene manipulation, often exercised *ex vivo*, has been used *in vivo*. Recombinant genes by themselves were shown to have been taken up and expressed in murine skeletal myofibers (Wolff et al., 1990; Ascadi et al., 1991; Davis et al., 1993) and cardiac myocytes (Leinwand and Leiden, 1991) following intramuscular injections. Gene expression is invariably low despite different delivery conditions and methods (Wolff et al., 1991). This approach lacks basis and evidence of gene integration and regulation.

4. Combinations

A more logical approach is to include viral or cellular transcriptional regulatory sequences to effect expression. In the prophylactic treatment of hemophilia A, a retroviral factor-VIII cDNA conjugate was used to induce secretion of the blood-clotting factor in athymic mice from transduced human skin fibroblasts implanted (Hoeben, 1995). Both adenoviral (Smith et al., 1993) and Herpes Simplex virus-derived (Miyanohara et al., 1992) vectors have similarly been used for *in vivo* transfer of factor IX cDNA to the liver. Although therapeutic levels of factor IX were obtained, the expression decayed in a few weeks, possibly due to immune response and gene inactivation (St. Louis and Verma, 1988).

Gene therapy with viral vectors has been developing rapidly, but judging from the results of cystic fibrosis and brain tumor clinical trials, it is still a young discipline (Rosenfeld and Collins, 1996; Alton and Geddes, 1994). Since the main thrust of this chapter is on myoblast transfer therapy (MTT), additional details of non-myoblastic single gene manipulations can be found in the books entitled "Gene Therapy - A Primer for Physicians" (Culver, 1996) "Somatic Gene Therapy" (Chang, 1994) and "Gene Therapy for Neoplastic Diseases" (Huber and Lazo, 1994).

5. Myoblasts

Although genetic ailments constitute less than 2% of all human diseases, far more currently incurable diseases are the result of inadequate genetic predisposition and/or haphazard interactions between multiple genes. Symptoms precipitate when a regulatory or a structural protein is either missing or malfunctional. Without knowing these defect(s) or how they can be corrected, tissue engineering will favor genome replacement rather than single gene(s) replacement. The cell knows more than we do.

Furthermore, for a gene therapy to be effective and efficient, transgene expression requires appropriate targeting into a specific cell type, integration onto a specific site on a specific chromosome, and regulation by factors that are the products of other genes. This chain of events involves numerous cofactors many of which are produced transiently during embryonic development but not in adulthood. This is where the approach of single gene manipulation is conceptually inadequate because it cannot provide these cofactors. In complex systems, one hardly knows what they are. Again only transfer of the whole normal genome can allow the orderly provision of these cofactors necessary for the transgene expression.

Finally secondary degenerative changes often accompany the primary protein defect. Additional structural and/or regulatory protein(s) are lost (**Fig. 1**). Even if single gene manipulation replaces the primary protein deficit, transduced cells still degenerate because of the secondary changes. These latter proteins can only be replaced by re-transcribing the normal genome inserted.

Myoblasts are muscle-building cells endogenous to the human body. Contained within the nucleus of each human myoblast is the normal genome with over 100,000 normal genes that determine cell normality and cell characteristics. Less than 10% of the gene actions is known. Myoblasts is the only somatic cell type in the body capable of natural cell fusion. Through this process, they insert their nuclei, and therefore all of the normal genes, into multinucleated myofibers of the host to effect genetic repair (**Fig. 2**).

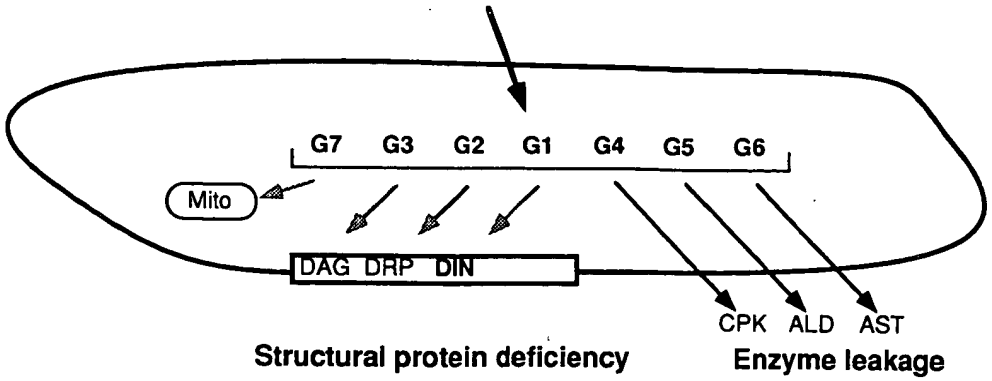

Fig. 1. Diagram of some of the known genetic factors in DMD muscle cells that differ from normal muscle cells. These include genes for membrane structural proteins that are decreased or absent in DMD, dystrophin (DIN), dystrophin-related-proteins (DRP) and dystrophin-associated glycoproteins (DAG), genes for enzymes elevated in serum levels of DMD patients, creatine phosphokinase (CPK), aldolase (ALD) and aspartate transaminase (AST), and genes for mitochondrial (Mito) differences.

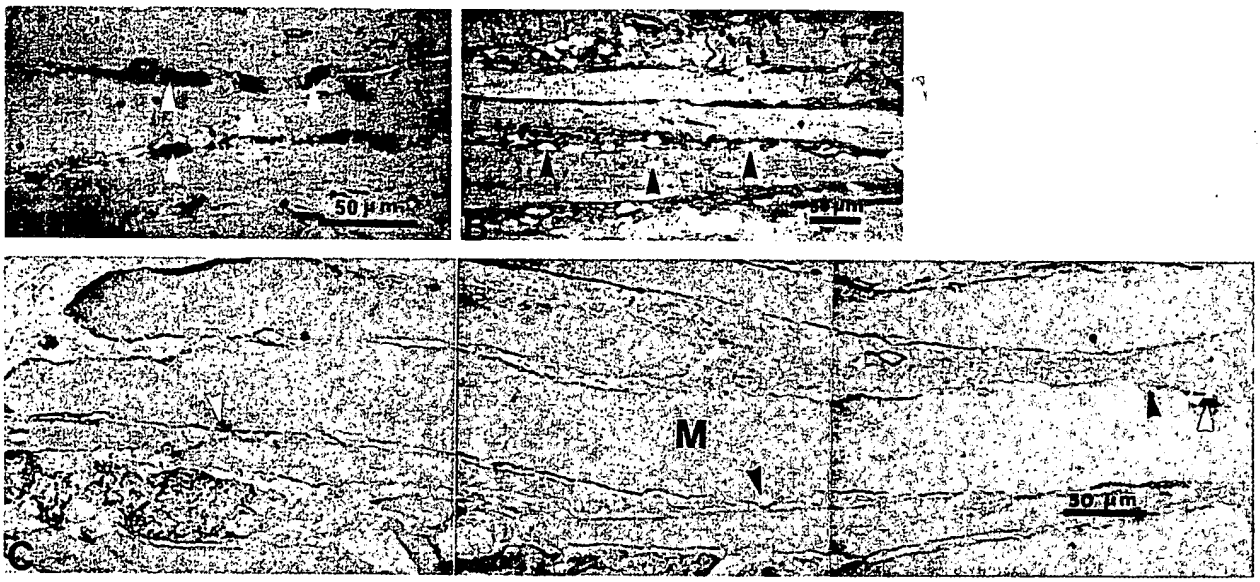

Fig. 2. Immunocytochemical localization of donor (stained, white arrowheads) and host (unstained, dark arrowheads) nuclei in longitudinal muscle sections. A and B are normal and dystrophic controls, respectively. C is from a dystrophic muscle 18 months after normal myoblast injection. A mosaic fiber (M) is demonstrated by the presence of both stained and unstained nuclei.

The transfer of genetic material and information occurs *in vivo*, with the myoblasts serving as the source and the vehicle to effect gene transfer.

Myoblasts are the only cells that divide extensively (Law et al., 1997a), migrate (Law et al., 1992), fuse naturally to form syncytia (Law et al., 1992), interiorizing major histocompatibility complex class I (MHC-1) antigens after fusion (Daar et al., 1984; Appleyard et al., 1985), and develop up to 50% of human body weight. Myoblast recipients need no more than two months of immunosuppression after MTT because mature myotubes and myofibers do not exhibit MHC-1 antigens (Daar et al., 1984; Appleyard et al., 1985). These combined properties render myoblasts superior for gene transfer. Being endogenous cells, myoblasts do not produce the adverse reactions of viral vectors.

II. Myoblast Transfer Therapy (MTT) technology

MTT is a platform technology of gene therapy and tissue engineering. The procedure consists of culturing large quantities of myoblasts from muscle biopsies of genetically normal human donors. Cultured myoblasts are

injected into patient's muscles while the patient is under general anesthesia. An immunosuppressant is administered following the procedure to minimize donor cell rejection.

The injection injury activates regeneration of host myofibers, allowing them to fuse with the injected myoblasts, thus forming genetically mosaic multinucleated myofibers (**Fig. 2**) (Law et al., 1988a,b, Chen et al., 1992). In addition, injected myoblasts fuse among themselves, forming genetically normal myofibers (Law et al., 1988a,b; Chen et al., 1992). Thus, MTT delivers the normal nuclei, the genetic software and hardware in total, into muscles of the genetically defective host, where the critical transgene is naturally and stably integrated, regulated and expressed.

Since the fusion process is a natural occurrence, there should not be any problem with specificities of integration, complementation, regulation, and expression of the normal genome inserted. It is not necessary to know which gene(s) is responsible for the defect. Abnormal gene identification is time-consuming and expensive. Furthermore, the injection of normal myoblasts directly into the host muscle eliminates any uncertainty of tissue targeting. Natural transcription of the normal genome within the donor nuclei following MTT ensures orderly replacement of any protein deficiency resulted from single gene defects or from haphazard polygenic interactions, much of which is unknown.

III. Muscular dystrophies: the testing ground

Muscular dystrophies are genetic diseases of progressive muscle degeneration. Debilitating and fatal, these hereditary degenerative diseases deprive their sufferers of a normal quality of life and life span. Duchenne muscular dystrophy (DMD) confines boys to wheelchairs by age 12 and claims their lives by 20. Second in prevalence only to cystic fibrosis, DMD afflicts one in every 3300 male births worldwide (Emery, 1991).

As with any hereditary degenerative disease, DMD treatment will require repairing degenerating cells and replenishing dead cells. MTT is unique in treating the muscular dystrophies in that it transfers the normal genome to repair degenerative myofibers and it provides normal cells to replenish degenerated myofibers. As such, MTT is a combined cell/gene therapy. Potentially, not only can MTT prevent further weakening, it can also increase muscle strength.

Like murine dystrophy, DMD serves as a disease model to test MTT as a cell/gene therapy in treating hereditary degenerative diseases. MTT is being developed to repair degenerating cells and to replenish degenerated cells of the muscles in all of the neuromuscular diseases affecting over one million people worldwide. In a broad sense MTT is tested for its feasibility, safety, and efficacy to integrate the normal human genome into genetically abnormal patients.

Since MTT incorporates all of the normal genes into the dystrophic myofibers to repair them, it should exert similar effects regardless of which gene is abnormal or which protein is missing. Accordingly, MTT should be as beneficial to the murine dystrophies showing laminin $\alpha 2$ mutation in the dy and dy^{2J} phenotypes (Sunada et al., 1995) as DMD showing dystrophin deletion (Hoffman et al., 1987), given adjustments from mouse to human.

IV. Animal experiments

To develop a treatment we need to know the pathogenesis of the disease. By comparing the electric (Law and Atwood, 1972; Law et al., 1976) and ultrastructural properties (Mokri and Engel, 1975; Law et al., 1983) of normal vs. dystrophic myofibers, the genetic defects in muscular dystrophy were established to result from membrane deterioration and dysfunction. Using a normal/dystrophic parabiotic mice model with cross-reinnervation of muscles, it was demonstrated that the dystrophic nervous system would support normal muscle development (Law et al., 1976; Saito et al., 1983). Without such knowledge, it would be imprudent to attempt strengthening dystrophic muscles with normal myogenic cell transfer.

Earlier developmental work of MTT consisted of two approaches that were disparate but complementary. These are the demonstration of safety and efficacy of transferring normal myogenic cells into the $dy^{2J}dy^{2J}$ dystrophic mice (Law, 1978; Law and Yap, 1979; Law, 1982) and the examination of the developmental fate of donor cells in normal mice (Partridge et al., 1978; Watt, 1982; Watt et al., 1982). The $dy^{2J}dy^{2J}$ dystrophic mice share a common gene defect of laminin $\alpha 2$ mutation with congenital muscular dystrophy, the most severe form of human dystrophies (Sunada et al., 1995).

It was not until 1989 that a study of MTT on mdx mice was first published (Partridge et al., 1989; Karpati et al., 1989). The majority of evidence in support of MTT safety and efficacy is derived from previous studies using the $dy^{2J}dy^{2J}$ mice (Law et al., 1988a,b; Chen et al., 1992; Law, 1978; Law and Yap, 1979; Law, 1982; Law et al., 1990b,d).

This was at a time when neither the golden retriever muscular dystrophy (GRMD) nor the xmd canine dystrophy was known. Dystrophic dogs are available to a few laboratories that have not produced any significant results with MTT (Kornegay et al., 1992).

Central to MTT is the correlation of genetic and phenotypic improvement at the cellular and at the whole muscle levels. These studies play an essential role in the elucidation of the mechanisms by which MTT exerts its beneficial effects on dystrophic muscles (Law et al., 1978; Law and Yap, 1979; Law, 1982; Law et al., 1988a,b; Chen et al., 1992; Partridge et al., 1989; Karpati et al., 1989; Law et al., 1990b,d).

The demonstration that cultured cells survived, developed and functioned *in vivo* after implantation into an organ of a genetically abnormal mammal bridges the gap between *in vitro* and *in vivo* cell biology. This was first achieved with myoblast transfer (Law et al., 1988a,b).

The foremost study in adult *dystrophic* mice was aimed at producing mosaic muscles containing normal, dystrophic and mosaic myofibers from the *normal* and *dystrophic* minced muscle mixes (Law, 1978). It focused on incorporating the "missing" gene and its product(s) into genetically defective cells through cell transplantation and natural cell fusion, the result of which is strengthened dystrophic muscles (Law, 1978) having a gene defect similar to human congenital muscular dystrophy (Sunada et al., 1995). The result contradicts the study of Partridge and Sloper (1977) who concluded, in transplanting normal minced muscles into normal hosts, that little or none of the regenerates was of donor origin. Eventually, fusion between host and donor myogenic cells of normal genotypes using skeletal muscle grafts were demonstrated with genotype marker (Partridge et al., 1978). Although this latter study did not involve dystrophic animals, it was inferred that MTT was a distinct development with potential applicability to hereditary myopathies.

In a later study, muscles of newborn normal mice were grafted into recipient soleus muscles of dystrophic mice. Results obtained 6 months after the grafting indicated that the grafts survived, developed, and functioned in the dystrophic environment. The regenerates had larger cross-sectional areas and more muscle fibers than the contralateral dystrophic solei. MTT increased the mean twitch tension of adult dystrophic muscles to that of the normal (Law and Yap, 1979). The concept of replenishing lost cells and repairing degenerative cells through the production of genetic mosaicism using MTT was firmly established (Law and Yap, 1979).

An important finding was that myoblasts cultured from muscle biopsies of adult normal rats could survive and develop in the original donor after implantation (Jones, 1979). MTT with cultured myoblasts became the logical development since myoblasts do not require neuronal and capillary connections to survive and develop, and since myoblasts can fuse to effect genetic repair.

A convenient way to obtain normal myoblasts in mice is through dissection of limb-bud mesenchyme of day-12 embryos. Dissected mesenchyme was surgically implanted into the solei of $dy^{2J}dy^{2J}$ mice. Host and donors were histocompatible. Contralateral solei served as controls. Six to seven months postoperatively, the myoblast-implanted solei exhibited greater cross-sectional area, total fiber number, better cell structure, and twitch and tetanus tensions than their contralateral controls (Law, 1982).

The incorporation and fusion of allogeneic muscle precursor cells *in vivo* were further explored using normal mice (Watt, 1982). The implants consisted of minced muscle mixes or newborn muscles (Watt et al., 1982; Watt et al., 1984; Morgan et al., 1988). It was confirmed that donor cells survived and developed in the host muscles, using electrophoretic analyses of glucose phosphate isomerases (GPI), the genetic markers to identify hosts vs. donor cells.

The use of cultured myoblasts with dystrophic mice eventually appeared. In the first study, primary myoblast cultures from limb-bud explants of normal mouse embryos were injected into the soleus muscles of histocompatible dystrophic hosts (Law et al., 1988,b). In the second study, clones of normal myoblasts were injected into the leg and intercostal muscles of histoincompatible hosts with cyclosporine-A (CsA) as a host immunosuppresant (Law et al., 1988a). Using GPI as genotype markers, donor myoblasts were shown to have fused among themselves, developing into normal myofibers. They also fused with dystrophic host myogenic cells to form mosaic myofibers of normal phenotype (Law et al., 1988a,b; Law et al., 1990a,c). These two mechanisms of genetic complementation were shown to be responsible for improvement in muscle genetics, structure, function and animal behavior of the test dystrophic mice (Law et al., 1988a,b; Law, 1978; Law and Yap, 1979; Law, 1982; Law et al., 1990b,d). Prolongation of the life-spans of the myoblast-injected dystrophic mice was demonstrated (Law et al., 1990b,d). The improvement persisted despite CsA withdrawal.

Morgan *et al.* (1988) reported the synthesis of trace amounts of phosphorylase kinase (PhK) in about 5% of the myoblast-injected muscles of the PhK-deficient mice. Although there have been frequent claims of supplying normal muscle precursor cells to alleviate hereditary myopathies, no evidence of any structural or functional improvement after transplantation was presented.

With the discovery that the absence of the gene product dystrophin is the cause of DMD (Hoffman et al., 1987) and mdx mouse dystrophy, a new biochemical marker became available to demonstrate MTT efficacy (Partridge et al., 1989; Karpati et al., 1989; Chen et al., 1992). With implantation of cultured normal myoblasts into muscles of immunosuppressed mdx mice, MTT was shown to convert mdx myofibers from dystrophin-negative to -positive (Partridge et al., 1989; Karpati et al., 1989). The study demonstrates biochemical improvements in the mdx mouse model, an additional evidence to confirm the efficacy of MTT.

Given the use of inbred mice that afford histocompatible MTT, the reality is that fully matched human donors and dystrophic recipients are rarely available. MTT would thus necessitate the inclusion of host immunosuppression to facilitate myoblast survival after transfer. Cyclosporine (Cy) is the most widely documented immunosuppressant in transplantation studies (Kahan and Bach, 1988). Availability of FK506 in the late 80's was limited (Starzl et al., 1991). Typically, host mice were primed 1 week with CsA injected subcutaneously every day at 50 mg/kg body weight before receiving myoblasts. The same CsA treatment continued for 6 months after MTT (Law et al., 1988b).

Aside from donor cell survival in an immunologically hostile host, cell fusion is the key to strengthening dystrophic muscles with MTT. To improve the fusion rate between host and donor cells, various injection methods aimed at wide dissemination of donor myoblasts were tested and compared. The goal was to achieve maximum cell fusion with the least number of injections.

The results indicate that delivery of myoblasts is best conducted by diagonal placement of needle into the host muscle with ejaculation of the myoblasts as the needle is withdrawn. This method of myoblast injection yields even and wide distribution of donor myoblasts with a high rate of cell fusion. Myoblasts injected perpendicular to myofiber orientation are partially distributed. Myoblasts injected longitudinally through the core of the muscles and parallel to the myofibers are poorly distributed (Law et al., 1994b). Thus myoblast injection method regulates cell distribution and fusion.

V. Clinical trials

Gene therapy encompasses interventions that involve deliberate alteration of the genetic material of living cells to prevent or to treat diseases (Kessler et al., 1993). According to this FDA definition, the first MTT on a DMD boy on February 15, 1990 marked the first clinical trial on human gene therapy (Hooper, 1990). In addition to fulfilling their primary muscle-building mission, the myoblasts served as the source and the transfer vehicles of normal genes to correct the gene defects of DMD. The protocol was approved by four institutional review boards (Law, et al., 1990c). Subjects and parents gave informed consents.

The safety and efficacy of MTT was assessed by injecting the left extensor digitorum brevis (EDB) muscle of a 9-yr-old DMD boy with about 8 x 10^6 myoblasts. Donor myoblasts were cloned from satellite cells derived from a 1 g rectus femoris biopsy of the normal, adoptive father. Cyclosporine was administered for three months at a dose of 5-7 mg/kg body weight divided into two daily oral doses.

Donor myoblasts survived, developed, and produced dystrophin in myofibers biopsied from the myoblast-injected EDB 92 days later. Dystrophin was not found in the contralateral sham-injected muscle. This first case suggested that MTT offered a safe and effective means for alleviating biochemical deficit(s) inherent in muscles of DMD (Law et al., 1990a).

A pioneering work (Anderson, 1990; see also Brenner, 1995; Karlsson, 1991) is often considered as the "first human gene therapy"; correction of the ADA deficiency study began on September 14, 1990 (Anderson, 1990), two months after the MTT correction of the DMD gene defect was published (Law et al., 1990a). In the ADA protocol, T cells from a patient with a severe combined immunodeficiency disorder (SCID) were transduced with functional ADA genes *ex vivo* and returned to the patient after expansion through culture. In the MTT protocol, primary culture of myoblasts derived from a muscle biopsy of a normal donor was injected into a muscle of the DMD subject to produce *in vivo* nuclear complementation. Both gene therapies utilize cell transplantation to treat diseases.

However, it is pointed out that the ADA protocol involved genetic modification and correction of the patients T cells with the *adenosine deaminase* gene whereas in the DMD protocol normal donor cells were used which were not genetically modified ex vivo.

Six years after the foremost MTT, dystrophin was found in the myoblast-injected muscle but not in the sham-injected muscle (**Figure 3,** Law, 1997). Six years is the longest period through which any gene therapy has sustained positive results. Despite cyclosporine withdrawal at 3 months after MTT, myofibers expressing foreign dystrophin were not rejected. This is because dystrophin is located in the inner surface of the plasma membrane, and because mature myofibers do not exhibit MHC-1 surface antigens. Not only has the result demonstrated MTT overall safety and efficacy in this single case, it also shows stability in the integration, regulation and expression of the inserted dystrophin gene. The presence of dystrophin in the myoblast-injected but not in the sham-injected muscle provided unequivocal evidence of the survival and development of donor myoblasts in the myoblast-injected muscle.

In a randomized double-blind study involving three subjects, myoblast-injected EDBs showed increases in tensions whereas sham-injected EDBs showed reductions (Law et al., 1991a,b). Both immunocytochemical staining and immunoblot revealed dystrophin in the myoblast-injected EDBs. Dystrophic characteristics such as fiber splitting, central nucleation, phagocytic necrosis, variation in fiber shape and size, and infiltration of fat and connective tissues were less frequently observed in these muscles. Sham-injected EDBs exhibited significant structural and functional degeneration and no dystrophin. Throughout the study, there was no sign of erythema, swelling or tenderness at the injection sites. Serial laboratory evaluation including electrolytes, creatinine, and urea did not reveal any significant changes before or after MTT.

To reconcile these positive results with less convincing ones (Gussoni et al., 1992; Huard et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Miller et al., 1992; Morandi et al., 1995; Tremblay et al., 1993), several issues need to be addressed. To begin with, the use of large quantities of pure live myoblasts is a pre-requisite of successful MTT. Except for one study (Law et al., 1992), there is no published pictorial evidence to substantiate the purity, myogenicity and viability of the injected myoblasts as claimed.

Myoblast cultures are usually contaminated with fibroblast overgrowth. MTT with such impure culture could lead to deposition of connective tissues rather than myofiber production. Culturing 50 billion pure human myoblasts for MTT from two grams of muscle biopsy has

only been reported by our team (Law et al., 1997a). Other teams work at ranges of hundreds of millions of myoblasts.

In most studies (Gussoni et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Miller et al., 1992; Morandi et al., 1995) myoblasts were transported frozen, chilled for over two hours from the site of harvest before being injected. Since myoblasts have a high metabolic rate, they could not have survived for two hours without significant nutrients, oxygen and proper pH, being closely packed in saline within a vial for transport. Determination of cell viability before MTT were not conducted in these studies. Our myoblasts were injected into the subject within minutes of harvest, at the same location without transport.

MTT studies that reported failure (Gussoni et al., 1992; Huard et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Miller et al., 1992; Morandi et al., 1995; Tremblay et al., 1993) subscribed to the fallacy of making 55 to 330 injections into a muscle the size of an egg, traumatizing indiscriminately the underlying nerves, muscle, and vasculature. These injection traumas boosted macrophage access and host immune responses (Guerette et al., 1995). They also induced fibrosis (Chen et al., 1988). Surviving myoblasts fused within three weeks in small mouse muscles (Chen et al., 1992). A nerve with multiple trauma could not regenerate soon enough through scar and connective tissues to innervate the newly-formed myotubes in a large human dystrophic muscle. Stabilization of muscle contractile properties in a similar situation is achieved by 60 days in the rat, and functional return is incomplete (Carlson, 1983). Non-innervated myotubes died within one week. Whatever few myotubes that developed in the unsuccessful MTT studies could not compensate for the traumatized myofibers.

In the study yielding positive results, 5 to 8 x 10^8 pure myoblasts were delivered with eight injections into the biceps brachii without nerve injury (Law et al., 1994a, 1997a). Contrarily, in another study, 55 sites, each 5 mm apart, distributed in 11 rows and 5 columns, were injected throughout the depth of each biceps of 5- to 9- year old boys (Mendell et al., 1995). This was repeated monthly for six months. Axonal sprouts, myotubes and neuromuscular junctions that take six weeks to mature (Fex and Jirmanova, 1969) were repeatedly traumatized by a total of 330 injections until the biceps, with or without myoblast/cyclosporine, were irreversibly damaged or destroyed. The result: no functional difference between myoblast- and sham-injected muscles (Mendell et al., 1995).

Once injected, the myoblasts are subjected to scavenger hunt by macrophages for up to three weeks. This is because myoblasts exhibit MHC-1 surface antigens (Friedlander and Fischman, 1979; Fang et al., 1994) that become absent after cell fusion. The latter occurs between one to three weeks after myoblast injection (Chen et al., 1992). An allowance in the number of injected myoblasts has to be made to satisfy the unavoidable scavenger process. As reflected in the small numbers of myoblasts injected in unsuccessful studies, it appears that either such allowance was not considered or that the teams were not able to produce larger quantities of pure myoblasts. Although myoblast loss can be minimized by down-regulating macrophage activity (Guerette et al., 1997), such additional compromisation of the host immune system may lead to higher risk of infection, since MTT subjects are already taking immunosuppressants.

The less successful MTT teams focused on immunosuppression to prevent T-lymphocyte proliferation and antibody production without overcoming the primary hurdle of providing enough pure and live myoblasts. A basic study indicates that cyclophosphamide did not permit myoblast engraftment in the mouse (Vilquin et al., 1995), and a MTT clinical trial was conducted without success using cyclophosphamide immunosuppression (Karpati et al., 1993). Cyclosporine (Law et al., 1990a) and potentially FK506 (Kinoshita et al., 1994) remain the immunosuppressants of choice for MTT. Results could have been more positive if either was employed in the study of Tremblay *et al.* (Huard et al., 1992; Tremblay et al., 1993).

All of these single muscle MTT studies had begun before the FDA established policies and regulations for cell/gene therapies. Our studies are the only ones that received permission for an investigational new drug application (IND) on MTT for treatment of multiple muscles. As a cell/gene therapy, all American MTT clinical trials must come under FDA purview.

Beginning with 8 million myoblasts into a small foot muscle, our team proceeded to test 5 billion cells into 22 leg muscles, 25 billion cells into 64 body muscles, and now 50 billion cells into 82 muscles (**Table 1**). With over 150 procedures having been conducted, the complete safety of the MTT procedure has been proven. There have been no adverse reactions or side effects.

Protocol	Myoblasts	Muscles	Subjects
1	8 million	1	11
2	5 billion	22	32
3	25 billion	64	40
4	50 billion	82	27

Table 1. Dose escalation protocols of MTT and the number of subjects receiving such procedures.

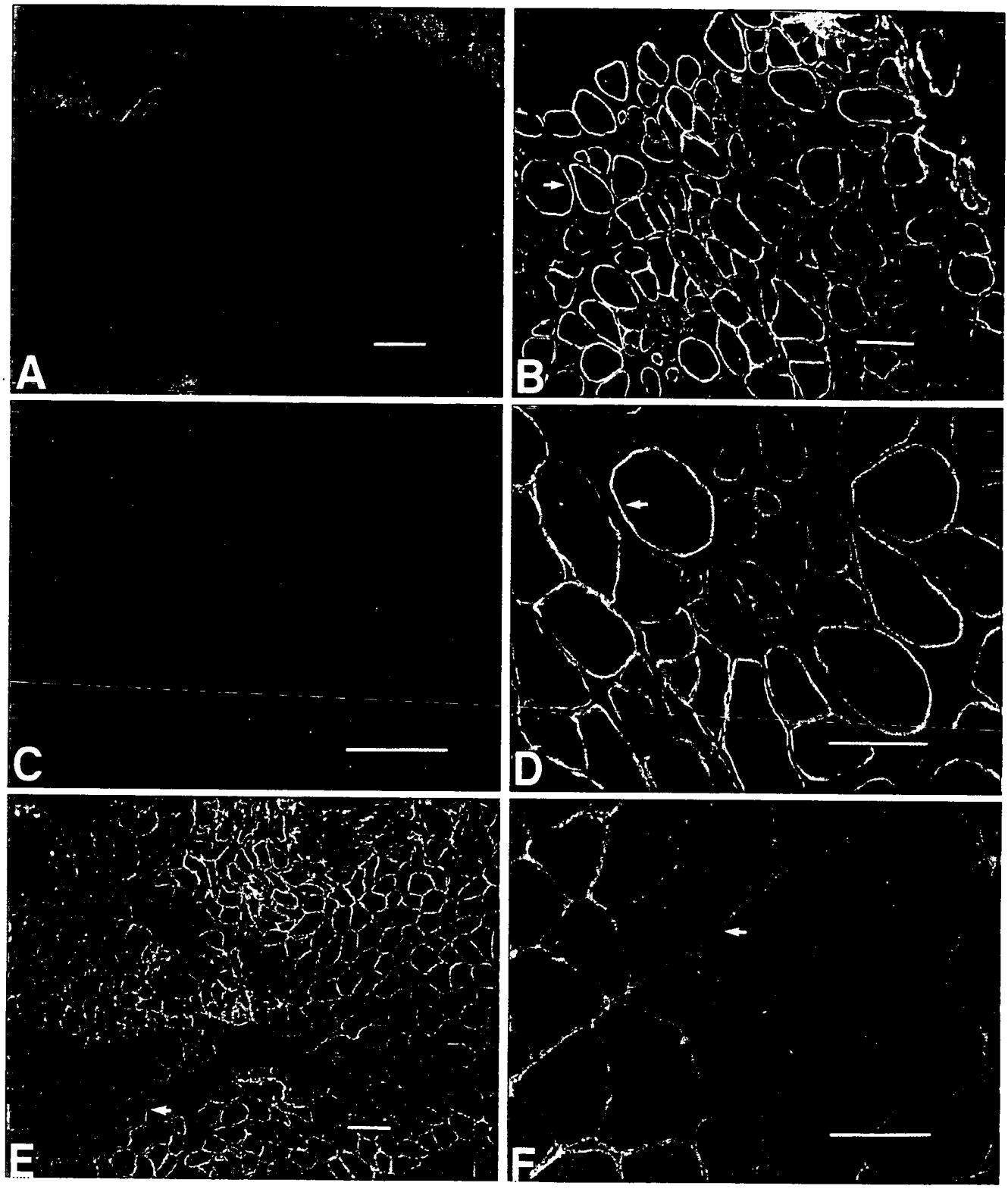

Fig. 3. Immunocytochemical demonstration of dystrophin in DMD muscles 6 yr after MTT. Dystrophin absent in sham-injected EDB muscle (**A,C**), but present in the contralateral myoblast-injected muscle (**B,D**). Dystrophin was immunocytochemically localized at the sarcolemma (arrows). Dystrophin demonstrated at low (**E**) and high (**F**) magnification in normal control muscle. Cross-section; bar = 100μm.

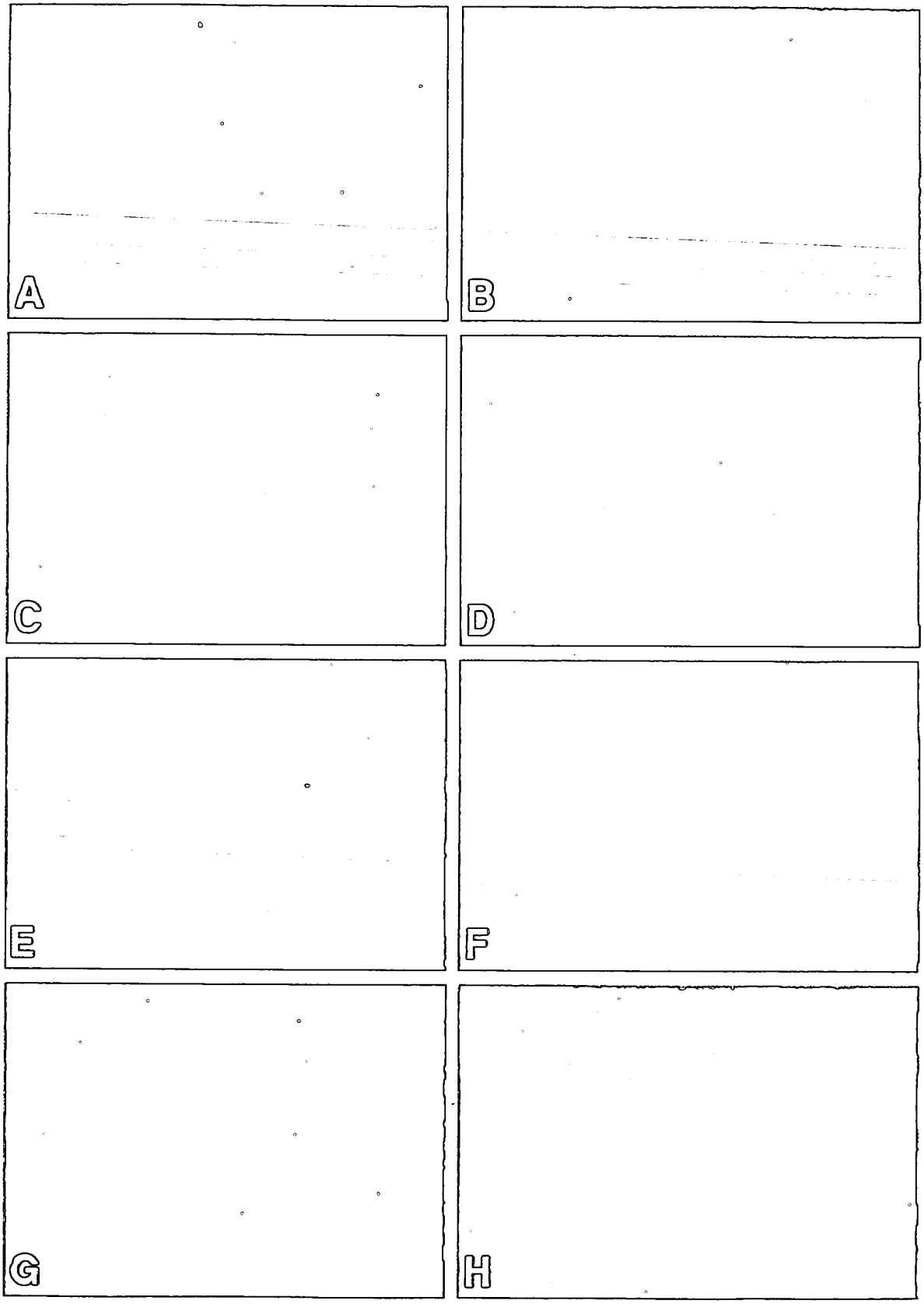

Fig. 4. Dystrophin immunocytochemistry showing the presence of dystrophin in (A) normal control and in (C,E,G) muscle biopsy specimens of three subjects. Dystrophin is absent in (B) Duchenne's muscular dystrophy control and in (D,F,H) contralateral biopsy specimens from the same subjects.

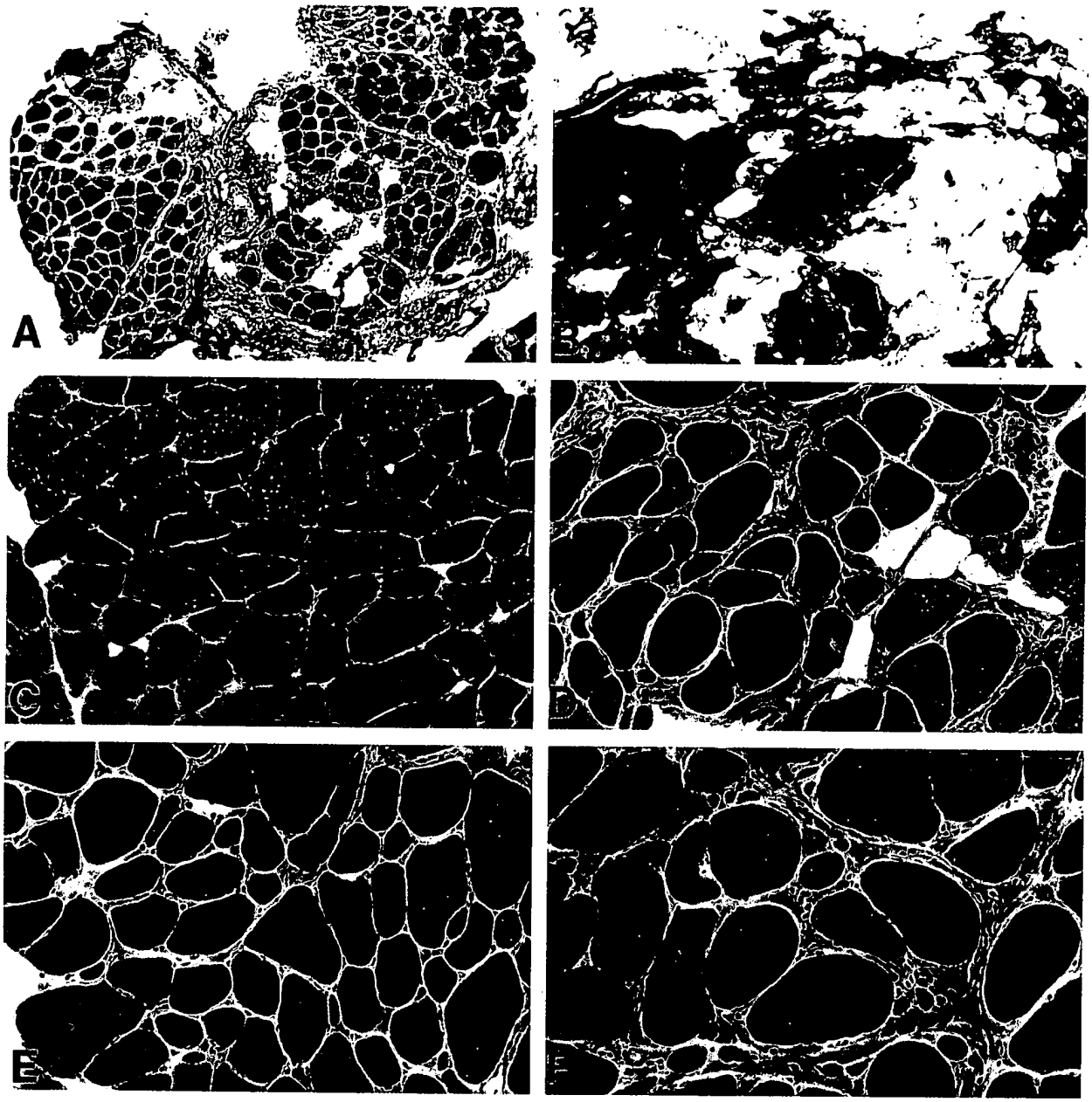

Fig. 5. (**A,C,E**) Three dystrophin-positive muscle biopsy specimens exhibit less dystrophic characteristics than the contralateral dystrophin-negative biopsy specimens (**B,D,F**). Dystrophic characteristics include increases in fat and connective tissue, fiber splitting, central nucleation, round and oval fibers.

The five billion myoblast cell protocol.

The 5-billion myoblast MTT protocol was tested in 32 DMD boys aged 6-14 yr. Through 48 injections, 5 billion myoblasts were transferred into 22 major muscles in both lower limbs under general anesthesia. Only four donors were histocompatible with their recipients. All subjects took cyclosporine for six months after MTT. More than 88% of the injected ankle plantar flexor muscles showed either increase in strength or no further deterioration at 9 months after MTT (Law et al., 1992, 1993).

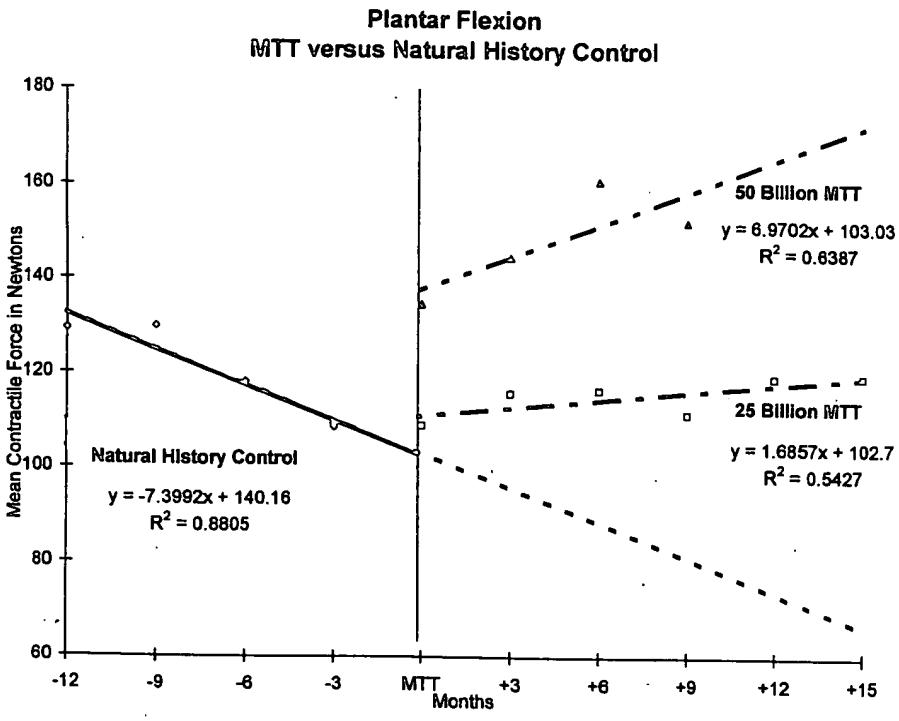

Fig. 6. Dose-dependent responses to MTT of plantar flexion with greater increase in maximum isometric force using the 50-billion MTT protocol than with the 25-billion MTT protocol. Both protocols show efficacy in strengthening the plantar flexion when compared to the natural history control.

Table 2. Percentage increases over a one-year natural history control in the maximum isometric force of the plantar flexor muscles at 3, 6, 9, 12, and 15 months after the administration of the 25-billion MTT protocol or the 50-billion MTT protocol.

	Months after MTT				
	3	6	9	12	15
25 Billion MTT (800 x 10^6 myoblasts)	9%	19%	31%	45%	61%
50 Billion MTT (1,200 x 10^6 myoblasts)	11%	23%	37%	53%	71%

The 25 billion myoblast cell protocol.

Under FDA purview, MTT is completing Phase II clinical trials on DMD. The whole body trial (WBT) consisted of injecting 25 billion myoblasts in two MTT procedures separated by 3 to 9 mo. Each procedure delivered up to 200 injections or 12.5 billion myoblasts to either 28 muscles in the upper body (UBT) or to 36 muscles in the lower body (LBT). A randomized double-blind portion of the study was conducted on the biceps brachii or quadriceps. Subjects took oral cyclosporine for 3 months after each MTT. One infantile facioscapulohumeral dystrophy and 40 DMD boys aged 6 to 16 received WBT in the past 36 months with no adverse reaction.

Nine months after MTT immunocytochemical evidence of dystrophin were demonstrated in 18 of the 20 DMD subjects biopsied (**Fig. 4**). Dystrophin positive sections showed less dystrophic characteristics than dystrophin-negative ones (**Fig. 5**). Forced vital capacity increased by 33.3% and maximum voluntary ventilation increased by 28% at 12 months after UBT (Law et al., 1997a).

Plantar flexion showed an increase of 45% in maximum isometric contraction force in 12 months in the DMD subjects when compared to the natural deterioration (**Fig. 6, Table 2**). Behavioral improvements in running, balancing, climbing stairs and playing ball were noted (Law et al., 1995; Law et al., 1996; Law et al., 1997a,c,d). Notable was a 16-yr-old DMD subject who continued to walk without assistance and capable of driving an automobile by himself.

50 Billion myoblast cell protocol.

The current study involves a one time injection of 50 billion myoblasts into 82 muscles with 179 skin punctures, approved by the FDA for subjects with DMD, Becker MD and Limb-girdle MD (Law et al., 1997d). Twenty-nine subjects who underwent this protocol have experienced no adverse reaction.

For the 22 DMD subjects aged 5 to 16, there was a significant increase in the maximum isometric force generated by the plantar flexor muscles at 3, 6, and 9 months after MTT (**Fig. 6, Table 2**).

This functional improvement is more pronounced with the 50-billion MTT than with the 25-billion MTT, indicating that it is dose-dependent. Thus, in the 25-billion MTT, 800 million myoblasts were injected into the plantar flexors, producing a mean 61% increase in force at 15-months after MTT. With the 50 billion MTT, 50% more myoblasts were injected, projecting a 10% greater increase in force at 15 months after MTT (**Fig. 6, Table 2**).

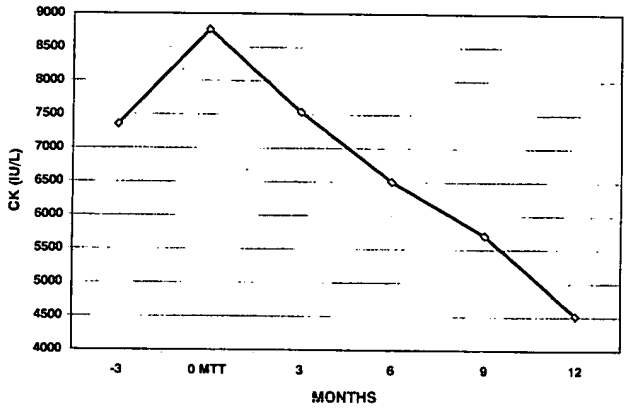

Fig. 7. Serum creatine kinase (CK) level of DMD subjects increased before MTT and decreased after MTT.

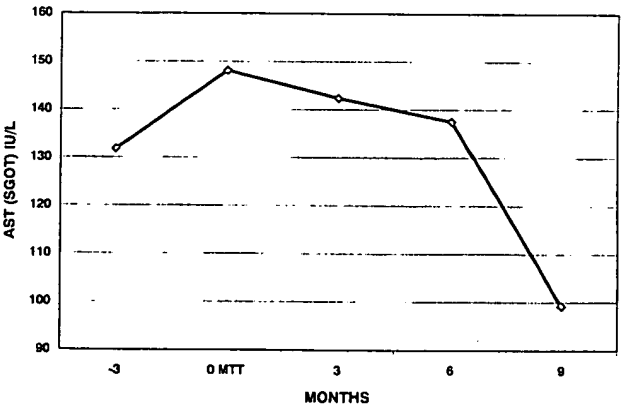

Fig. 8. Serum aspartate aminotransferese (AST) level of DMD subjects increased before MTT and decreased after MTT.

Elevated serum creatine kinase (**CK**) has traditionally been used to diagnose muscle degeneration, notable in DMD (Heyck et al., 1966). The 22 DMD subjects, mean ages 10.7-yr-old and, median age 9.9 yr-old, showed a 19.3% increase in serum CK within 3 months before MTT (**Fig. 7**). This trend was reversed after MTT, and the serum CK declined at a steady rate of 48.7% over 12 months. This result provides strong evidence that MTT repairs muscle cell membrane leakage of enzymes. This contention is further substantiated by similar findings with another muscle enzyme AST, aspartate aminotransferase (**Fig. 8**).

The breakthrough came when a 29-yr-old Becker MD (BMD) subject began to walk, with his hands being held, beginning at 2.5 months after the 50-billion MTT. He had previously been diagnosed repeatedly with BMD. He had been non-ambulatory and required the use of a wheelchair for over four years as documented in his medical record. He began walking with assistance a total of eight steps at 3 months after MTT. This ability increased with time, now reaching 60 steps at eight months after MTT. He began to stand and walk with his crutches at four months after MTT (**Fig. 9**).

VI. Future perspectives

As an universal gene transfer vehicle with which the entire human genome can be integrated into patient's muscles, myoblasts have shown promise in studies of the following diseases:

Cardiomyopathy. Labeled cultured myoblasts engrafted and formed structures resembling desmosomes, intercalated discs, fascia adherents junctions, and gap junctions in myocardia of dogs (Chiu et al., 1995), rats (Murry et al., 1996) and mice (Robinson et al., 1996) when MTT was delivered intramuscularly (Chiu et al, 1995; Murry et al., 1996) or intraarterially (Robinson et al., 1996). Donor muscle regenerates exhibited cardiac-like properties such as central nucleation (Chiu et al., 1995), fatigue resistance, slow twitching, and were capable of twitch and tetanus contractions when stimulated (Murry et al., 1996). Similar results were obtained when cardiomyocytes were injected in dystrophic mice and dogs (Koh et al., 1995), rats (Li et al., 1996) and swine (Van Meter et al., 1995). These findings, together with established MTT safety, pave the way to MTT clinical trial in treating myocardial degeneration and dysfunction.

Insulin-resistant diabetes mellitus. Commonly known as Type II diabetes, this disease is genetically predisposed and afflicts 90% of the diabetic population. Virtually all identical siblings of these patients develop the disease, and the genetic defect can be traced to the GLUT4 gene deletion. The major sequela of insulin resistance is decrease muscle uptake of glucose, due to the moderate decrease in insulin receptors on muscle cell surface. Conceptually MTT can add genetically normal myofibers with normal insulin receptors. It can also genetically repair the patients' myofibers and produce normal insulin receptors on the heterokaryons. Basic research is need to test this hypothesis on diabetic rats.

Fig. 9. First muscular dystrophy subject ever to walk after wheelchair bound for years.

(A). The 29-yr-old BMD subject had been wheelchair-bound for over 4 years.

(B,C,D,E) He began to walk with his hands held at 2.5 months after the 50-billion MTT.

(F) At 4 months after MTT, he was able to walk on crutches for about 20 steps.

Bone/cartilage degeneration. During embryonic development, mesenchymal progenitor cells differentiate into myoblasts, osteoblasts, chondrocytes and adipocytes under controls of various regulatory factors. Ectopic bone formation in muscle has been achieved through implantation of bone morphogenetic protein (BMP). BMP-2 was shown to convert the differentiation pathway of clonal myoblasts into the osteoblast lineage (Katagiri et al., 1994). This opens new ways to treat conditions of bone degeneration such as the degeneration of tooth pulp, hip, bone/joint, and long bone fractures. Given the ability to mass-produce myoblasts that can be transformed into osteoblasts, and potentially chondrocytes, the difficulty of proliferating osteoblasts and chondrocytes can be overcome. Cultured autologous chondrocytes can be used to repair deep cartilage defects in the femorotibial articular surface of the human knee joint (Brittberg et al., 1994).

The use of normal or transduced myoblasts as the source and vehicles for gene delivery has found application in the potential treatments of restenosis (Morishita et al., 1995), soft tissue deformities (Teboul et al., 1995), hemophilias (Dai et al., 1992; Yao et al., 1994), anemia (Hamamori et al., 1994), muscle trauma (Almeddine et al., 1994), human growth hormone deficiency (Barr and Leiden, 1991) and allograft rejection (Lau et al., 1996). MTT has produced a new frontier in medicine.

VII. Our vision

MTT implementation can benefit from the development of the following programs (Law, 1994):

Controlled cell fusion. It will be useful to be able to control, initiate or facilitate cell fusion once myoblasts are injected. This is to minimize loss of myoblasts from macrophages whose presence is unavoidable if the patient is to have some immune protection.

As the myoblasts are injected intramuscularly into the extracellular matrix, injection trauma causes the release of basic fibroblast growth factor (bFGF) and large chondroitin-6-sulfate proteoglycan (LC6SP). These latter growth factors stimulate myoblast proliferation. Unfortunately, they also stimulate the proliferation of fibroblasts that are already present in increased amount in the dystrophic muscle. That is why it is necessary to inject as pure as possible fractions of myoblasts in MTT without contaminating fibroblasts.

Controlled cell fusion can be achieved by artificially increasing the concentration of LC6SP over the endogenous level. In addition, insulin or insulin-like growth factor I (IGF-1) may facilitate the developmental process, resulting in the formation of myotubes soon after myoblast injection. Enhanced fusion of myoblasts into myotubes had been achieved with the use of PDO98059 (Coalican et al., 1997) and ED2+ macrophages conditioned medium (Massimino et al., 1997). The use of these compounds in the cell culture medium and in the injection medium will likely lead to greater MTT success.

Superior cell lines. These cell lines should be highly myogenic, nontumorigenic, nonantigenic, and will develop very strong muscles. The superior cell lines will bypass the use of immunosuppressant, and will provide a ready access for patients who do not have a donor. A unique property of myoblasts is their loss of MHC-I antigens soon after they fuse. The immunuosuppression period depends on how soon the myoblasts lose their MHC-I antigens after MTT. Even more ideal is the establishment of a myoblast cell line in which MHC-I antigens are absent. In human myoblasts cultured from normal muscle biopsies, some 91.7% of the myoblasts reacted with MHC-I MAb (monoclonal antibodies). The remaining 8.3% of the myoblasts, that were negative for MHC-I antigen expression were successfully separated by cytofluorometry. The lack of MHC-I antigens on these latter myoblasts may enhance survival of these myoblasts in recipients after MTT (Fang et al., 1994).

Automated cell processors. The great demand for normal and transduced myoblasts, the labor intensiveness and high cost of cell culturing, harvesting and packaging, and the fallibility of human imprecision will soon necessitate the invention and development of automated cell processors capable of producing huge quantities of viable, sterile, genetically well-defined and functionally demonstrated biologics.

This invention will be one of the most important offspring of modern day computer science, mechanical engineering and cytogenetics. The intakes will be for biopsies of various human tissues. The computer will be programmed to process tissue(s), with precision controls in time, space, proportions of culture ingredients and apparatus maneuvers. Cell conditions can be monitored at any time during the process and flexibility is built-in to allow changes. Different protocols can be programmed into the software for culture, controlled cell fusion, harvest and package. The outputs supply injectable cells ready for cell therapy or shipment. The cell processor will be self-contained in a sterile enclosure large enough to house the hardware in which cells are cultured and manipulated.

Transport medium. A transport medium that can sustain the survival and myogenicity of myoblasts in package for up to four days will allow the cell packages to be delivered to remote points of utilization around the world. **Fig 10** shows the effectiveness of such a medium developed in our foundation. Fifty billion myoblasts can be shipped at 4° C for four days with 90% viability.

Cell banks. The automated cell processors will constitute only a part of the cell banks. The current thought is to obtain donor muscle biopsies from young adults aged 8 to 22 to feed the inputs. Each donor has to undergo a battery of tests that are time-consuming and expensive. From the test results and from the donor's physical conditions, one can determine if the donor cells are genetically defective or infected with viruses and/or bacteria.

Human fetal tissues can potentially provide greater supplies of cells. However, aside from ethical issues surrounding abortion, it is difficult to determine the genetic normality of the cells. Muscle primordia of fetus derived from *in vitro* fertilization of genetically well-defined background may be an alternative. Sperm and ova can be recovered from healthy individuals that are known for their muscle strength and mass. *In vitro* fertilization will be followed by embryo culture to a specific developmental stage (day 26 to day 56 gestation) of the embryos. The muscle primordia that are rich in myoblasts can then be dissected out to feed the automated cell processors.

VIII. Conclusion

This chapter describes the landmark development of the first gene therapy study in humans. Through natural cell fusion, myoblasts transfer the human genome into dystrophic muscle cells to effect phenotype repair. The innovative cell transplantation procedure also revitalizes the degenerative organ by providing living cells of normal genotype to replenish cell loss. The result is potentially a new form of medicine. The conceptual approaches of single gene transfer and myoblast transfer toward treatment of hereditary degenerative diseases are compared.

As more scientists continue to recognize myoblasts as a stable source of genes and a safe and efficient gene transfer vehicle, MTT application will extend far beyond the treatment of neuromuscular diseases. This chapter provides insights to guide future development of MTT in battling against genetic and acquired diseases that presently have only diagnoses but no treatment.

Acknowledgment

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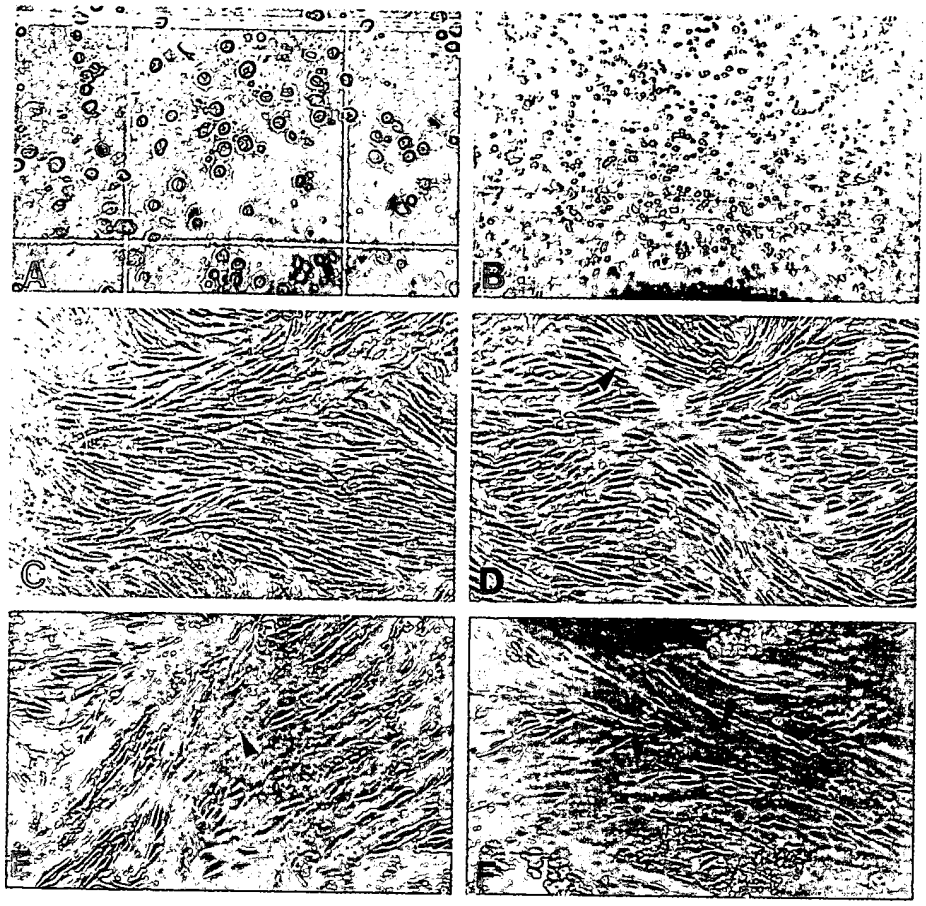

Fig. 10. Transport medium effectiveness as demonstrated by myoblast survival and myotube formation. (A). Myoblasts before a 50-billion MTT showing 99% viability using the vital stain erythrocin B,1% at pH 7.23. (B). Myoblast left-over from a 50-billion MTT maintained in the transport medium for 4 days at 4° C and stained with erythrocin B. The sample showed 90% viability. (C). Cells in B were put back into culture for 2 days before feeding fusion medium. (D). Cells in C in fusion medium for 1 day, showing myoblast fusion (arrow). (E). Cells in C in fusion medium for 2 days, showing myotubes (arrow). (F). Cells in C in fusion medium for 5 days, showing extensive myogenic capability in myotube formation (arrows).

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Commentary

FIRST HUMAN MYOBLAST TRANSFER THERAPY CONTINUES TO SHOW DYSTROPHIN AFTER 6 YEARS

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☐ **Keywords — Myoblast transfer; Dystrophin; Gene Therapy; Duchenne muscular dystrophy.**

Gene therapy encompasses interventions that involve deliberate alteration of the genetic material of living cells to diagnose, prevent, or treat disease (28). The administration of cells that have undergone ex vivo genetic manipulation is considered a combination of somatic cell therapy and gene therapy (15). Although the majority of human gene therapy trials to date have used this combination approach, gene therapy products have also been administered directly to subjects to modify cells in vivo (28). One of these latter gene therapy products is myoblasts, and because myoblasts are cells, myoblast transfer therapy (MTT, U.S. Patent # 5,130,141) is a combination of somatic cell therapy and gene therapy designed to effect gene transfer in vivo.

The use of myoblasts as gene transfer vehicles dates as early as 1978 (31,32,42). In mammals, myoblasts are the only cells that divide extensively, migrate, fuse naturally to form syncytia, lose MHC-1 antigens soon after fusion, and develop to occupy 50% of the body weight in humans. These combined properties rendered myoblasts ideal for gene transfer. Expression of a foreign gene requires appropriate integration and regulation involving numerous cofactors, many of which are transient during embryonic development (33). Natural transduction of the normal genome following MTT will ensure replacement of dystrophin and related proteins involving polygenomic interaction in Duchenne muscular dystrophy (DMD).

The first published conceptual approach, dated 1978, to incorporate the "missing" gene and its product(s) into genetically defective cells of adult mammals utilized cell transplantation and natural cell fusion to strengthen hereditary degenerative muscles (31). By 1979, normal myogenic cell transfer was shown to have improved the function of single muscles of the dystrophic mice to normal (42). MTT has since been shown to have improved the cell genetics, structure, muscle function, animal behavior, and life span of the $dy^{2J}dy^{2J}$ dystrophic mice (32,40,41). With the discovery in 1987 of dystrophin, the absence of which characterizes DMD and mdx mouse dystrophy, a new biochemical marker became available to demonstrate MTT efficacy in DMD and mdx mice (8,47). These pioneering animal experiments led to clinical trials on DMD boys.

The first MTT on a DMD boy occurred on February 15, 1990. It was the first human gene therapy clinical trial (23). Cultured myoblasts capable of natural cell fusion were used as vehicles to deliver their complete normal genomes into DMD myofibers to repair genetic defects. As a cell therapy, MTT was to replenish the degenerated myofibers with normal ones developed through fusion among donor myoblasts (40,41). In addition to fulfilling their primary muscle-building mission, the myoblasts served as the source and the transfer vehicles of normal genes to correct the gene defects of DMD.

The study protocol received approval from 1) the Institutional Review Board of the University of Tennessee Memphis (UTM); 2) the Scientific Advisory and Administrative Committees of the Clinical Research Center in

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UTM; 3) the LeBonheur Children's Medical Center Institutional Review Board; and 4) the UTM Data and Safety Monitoring Board.

The safety and efficacy of MTT was assessed by injecting the left extensor digitorum brevis (EDB) muscle of a 9-yr-old adopted boy having DMD with about 8×10^6 myoblasts. Donor myoblasts were cloned from satellite cells derived from a 1 g rectus femoris biopsy specimen of the normal, adoptive father. The only immunosuppressive agent administered was cyclosporine at a dose of 5–7 mg/kg body weight divided into two daily oral doses. The dosage was varied to maintain serum trough concentrations in the range of 100–150 ng/mL for 90 days.

Donor myoblasts survived, developed, and produced dystrophin in myofibers biopsied from the myoblast-injected EDB 92 days later. Dystrophin was not found in the contralateral muscle sham-injected with an equal volume (0.4 mL) of the carrier solution. This first case suggests that MTT offered a safe and effective means for alleviating biochemical deficit(s) inherent in muscles of DMD (34).

An often-asked question is how long can the myoblasts survive if they succeed in engrafting. To this end we have rebiopsied the myoblast- and sham-injected EDB muscles of the referenced world's first subject 6 yr after receiving MTT. Throughout this period these muscles did not receive any additional myoblasts or any other treatment.

Figure 1A and C shows the absence of immunocytochemical dystrophin in the control muscle sham-injected with 0.4 mL of the carrier saline. Dystrophin was present in the contralateral, myoblast-injected muscle (Fig. 1B and D). It was immunocytochemically localized at the sarcolemma using the method of Bonilla et al. (6). Although over 95% of the biopsied myofibers exhibited dystrophin, they are irregular in shape and size. Many appeared oval and small when compared to the polygonal, regularly sized normal control myofibers that are closely packed with little intercellular connective tissues (Fig. 1E and F).

At no time during the 6 yr after myoblast injection was there any sign of erythema, swelling, tenderness, or inflammation at the injection sites. Subsequent to this initial MTT in 1990, the subject received an additional 5×10^9 myoblasts in 22 major muscles in both legs in 1991, and another 25×10^9 myoblasts in 64 major muscle groups in both upper and lower bodies in 1994. In neither case were myoblasts administered close to the feet where the EDB muscles are located.

The result indicates that donor myoblasts survived, developed, and produced dystrophin within 92 days after MTT (34), and that the dystrophin-positive myofibers survived almost 6 yr. A reasonable assumption is that dystrophin prevented these fibers from undergoing degeneration, but such contention still awaits more definitive evidence. Despite the presence of dystrophin, the small size and oval shape of some of these myofibers suggest that they might not function normally, possibly due to atrophy as a result of reduced muscle activities. Nonetheless, the production of the structural protein dystrophin 6 yr after MTT provides confirmatory evidence of the correction of the primary gene defect of DMD. MTT appears to be a safe and effective gene therapy capable of producing long-term effect. Six years is the longest period for which any gene therapy has been followed with positive result.

One possible explanation of the presence of dystrophin in the myoblast-injected muscle is that reverse mutation might have occurred early during the development of this dystrophic muscle, thereby producing a high proportion of dystrophin-positive fibers. However, reverse mutation causing dystrophin production in adjacent myofibers occupying over 95% of the muscle biopsy with over 120 myofibers as shown in Fig. 1B has not been reported. Reverse mutation as published in the literature has accounted for no more than six dystrophin-positive fibers in similar sections.

To reconcile the current positive result with the less convincing ones reported by others (20,24,26,43–45,51) several issues need to be addressed.

To begin with, the use of large quantities of pure live myoblasts is a prerequisite of successful MTT. Besides Law's study (37), there is no published *pictorial* evidence to substantiate the purity, myogenicity, and viability of the injected myoblasts as claimed.

Myoblast cultures are usually contaminated with fibroblast overgrowth. MTT with such impure culture could lead to deposition of connective tissues rather than myofiber production. Culturing 25 billion pure human myoblasts for MTT from 2 g of muscle biopsy has only been reported by Law et al. (39). Other teams work at ranges of hundreds of millions of myoblasts.

In most studies (20,26,43–45) myoblasts were transported frozen, chilled, or at room temperature for about 2 h from the site of harvest before being injected. Because myoblasts have a high metabolic rate, they could not have survived for 2 h without significant nutrients, oxygen, and proper pH, being closely packed in saline within a vial for transport. Our myoblasts were injected into the subject within 30 min of harvest, at the same location without transport.

MTT studies that reported failure (20,26,43–45) subscribed to the fallacy of making 55 to 330 injections into a muscle the size of an egg, traumatizing indiscriminately the underlying nerves, muscle, and vasculature. These injection traumas boosted macrophage access and host immune responses (19). They also induced fibrosis

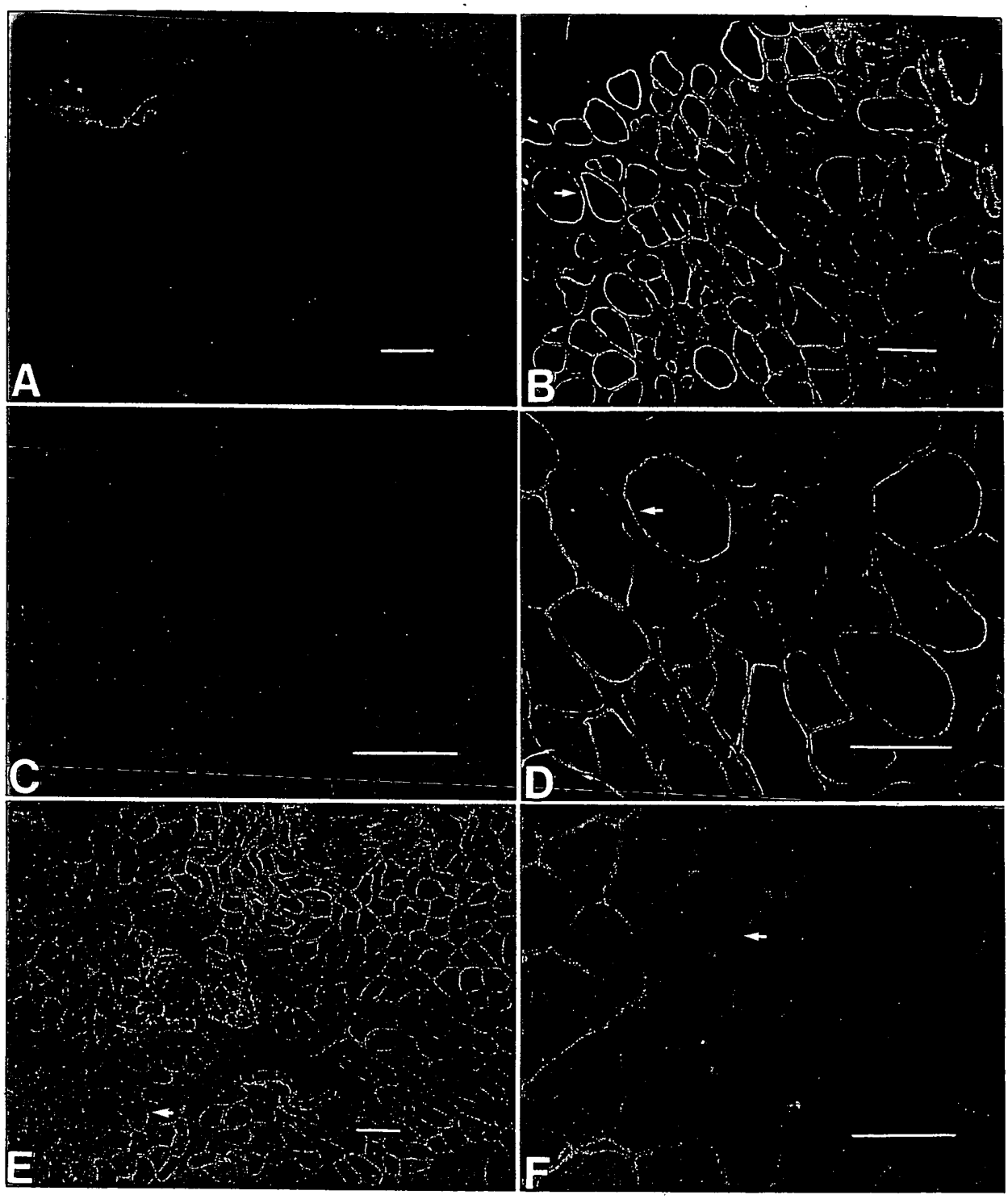

Fig. 1. Immunocytochemical demonstration of dystrophin in DMD muscles 6 yr after MTT. Dystrophin absent in sham-injected EDB muscle (A,C), but present in the contralateral myoblast-injected muscle (B,D). Dystrophin was immunocytochemically localized at the sarcolemma (arrows). Dystrophin demonstrated at low (E) and high (F) magnification in normal control muscle. Cross-sections; bar = 100 μm.

(9). Surviving myoblasts fused within 3 wk in small mouse muscles (8). A nerve with multiple trauma could not regenerate soon enough through scar and connective tissues to innervate the newly formed myotubes in a large human dystrophic muscle. Stabilization of muscle contractile properties in a similar situation is achieved by 60 days in the rat, and functional return is incomplete (7). Noninnervated myotubes died within 1 wk. Whatever few myotubes that developed in the unsuccessful MTT studies (20,26,43–45) could not compensate for the traumatized myofibers.

With eight injections we delivered 5 to 8 × 10^8 pure myoblasts into the biceps brachii without nerve injury (39). Contrarily, in Mendell's study, 55 sites, each 5 mm apart, distributed in 11 rows and 5 columns, were injected throughout the depth of each biceps of 5- to 9-yr-old boys (43). This was repeated monthly for 6 mo. Axonal sprouts, myotubes, and neuromuscular junctions that take 6 wk to mature (17) were repeatedly traumatized by a total of 330 injections until the biceps, with or without myoblast/cyclosporine, were irreversibly damaged or destroyed. The result—no functional difference between myoblast- and sham-injected muscles (43). How myoblasts escaped rejection and produced donor-derived dystrophin in two nonimmunosuppressed patients (#6 and #12) in that study cannot be explained. One would predict immune rejections and failure considering monthly repetition of large numbers of injections of histoincompatible myoblasts without cyclosporine.

Once injected, the myoblasts are subjected to scavenger hunt by macrophages for up to 3 wk. This is because myoblasts exhibit major histocompatibility complex class I (MHC-1) surface antigens (16,18) that eventually become absent after cell fusion (12). The latter occurs between 1 to 3 wk after myoblast injection (8). An allowance in the number of injected myoblasts has to be made to satisfy the unavoidable scavenger process. As reflected in the small numbers of myoblasts injected in unsuccessful studies, it appears that either such allowance was not considered or that the teams were not able to produce larger quantities of pure myoblasts.

The less successful MTT teams focused on immunosuppression to prevent T-lymphocyte proliferation and antibody production without overcoming the primary hurdle of providing enough pure and live myoblasts. A basic study indicates that cyclophosphamide did not permit myoblast engraftment in mouse (52). Without this prior knowledge, a MTT clinical trial was conducted without success using cyclophosphamide immunosuppression (26). Cyclosporine (34) and potentially FK506 (29) remain the immunosuppressants of choice for MTT. Results could have been more positive if either was employed in the study of Tremblay et al. (24,51).

All gene therapies involving viral vectors are still in Phase I clinical trials to determine safety. The gene therapy protocol for correction of adenosine deaminase (ADA) deficiency began on September 14, 1990 (2,3,11), 2 mo after MTT correction of DMD gene defect was published (34). In the ADA protocol, T cells from a patient with a severe combined immunodeficiency disorder (SCID) were transduced with functional ADA genes ex vivo and returned to the patient after expansion through culture. In the MTT protocol, primary culture of myoblasts derived from a muscle biopsy of a normal donor was injected into a muscle of the DMD subject to produce in vivo nuclear complementation. Both gene therapies utilize cell transplantation to treat diseases.

Today, the five subjects who underwent two ADA protocols with multiple readministration continue to be on enzyme replacement therapy, polyethylene glycol-conjugated ADA, and it is questionable as to whether the ADA gene therapy is effective. Over 130 DMD boys have received MTT with no adverse reactions since February 15, 1990. Functional, histologic, and biochemical improvements of DMD muscles have been reported (20,24,34–38,51). MTT on DMD is the most advanced form of gene therapy in which the myoblasts that are endogenous to the human body are used to effect gene transfer. We are conducting the first and only FDA-permitted Phase II MTT clinical trial on DMD under an investigational new drug application (IND), injecting 64 muscles of the upper and lower bodies with 25 billion myoblasts.

The foremost correction of primary gene defect in human (34), published on July 14, 1990, fueled immense enthusiasm of using normal and transduced myoblasts as gene delivery vehicles (5,31,32,40–42). In addition to clinical trials on Duchenne muscular dystrophy (16,20,24,26,35–38,43–45), MTT has found potential application in cardiomyopathy (10,30,49), restenosis (46), diabetes mellitus (48), human growth hormone deficiency (4,14), hemophilias (13,53), anemia (21), bone degeneration (27), Parkinsonism (25), soft tissue augmentation (50), and muscle trauma (1). Undoubtedly MTT has produced a new frontier in medicine with promise of treating hereditary and acquired diseases.

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Appendix D

AUGMENTATION OF BODY PARTS
(SOFT TISSUE)

Fig. 1. (A) Subject J.B. received about 600 million myoblasts at the right deltoid muscle which showed enlargement in size and provided shape and consistency when conformed to

(B) in which the deltoid muscle received placebo. The subject suffers from muscular dystrophy. Pictures were taken six months after myoblast transfer.

(C) Another muscular dystrophy subject showed an increase in size, shape and consistency of the biceps brachii muscle at six month after receiving 500 million myoblasts.

Apparently myoblasts can survive and develop when injected into adipose tissue including the breast, hip, check, buttock, etc. (Satoh et al, 1992).

Furthermore, myogenic cells such as myoblasts can be converted in fat cells to augment the size, shape and consistency of soft-tissues (Teboul et al. 1995).

It is Fig. 1, data unpublished, which shows the actual enablement.